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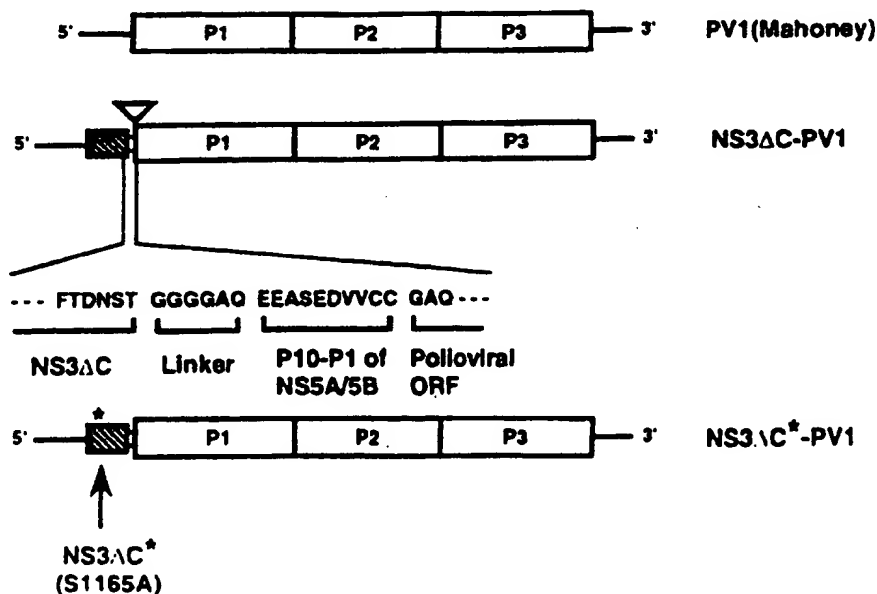
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(54) Title: **HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE AND A USE THEREOF**



(57) Abstract

The recombinant poliovirus which comprises poliovirus, hepatitis C virus (HCV) protease NS3 and target sites of the hepatitis C virus protease NS3, and needs the HCV protease activity, is produced, because efficient culture system for drugs against hepatitis C virus (HCV) which is the major etiologic agent of non-A, non-B hepatitis is lack.

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**HEPATITIS C SURROGATE VIRUS FOR TESTING THE ACTIVITY  
OF HEPATITIS C VIRUS PROTEASE, A RECOMBINANT GENE  
AND A USE THEREOF**

**BACKGROUND OF THE INVENTION**

5           **Field of the Invention**

The present invention relates to a hepatitis C virus (HCV), more particularly, is related to a hepatitis C surrogate virus which comprises poliovirus genome, HCV protease and its target site, and is suitable for testing efficacy of anti-HCV drugs and screening the same, a recombinant gene and a use thereof.

10           **Description of the Related Arts**

There are known some types of viruses which cause viral hepatitis. Hepatitis A virus is an RNA virus having a diameter of 27 nm and causing epidemic hepatitis through oral infection. Hepatitis B virus is a DNA virus having a diameter of 42 nm and causing hepatitis through blood infection. A hepatitis virus which does not belong to any of the above types was called non-A, non-B hepatitis virus.

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis (Alter *et al.*, *N. Engl. J. Med.* 321, 1494-1500, 1989; Choo *et al.*, *Science*, 244, 359-362, 1989; Kuo *et al.*, *Science* 244, 362-364, 1989). This virus has been implicated in liver cirrhosis and hepatocellular carcinoma (Bruix *et al.*, *Lancet* ii, 1004-1006, 1989; Saito *et al.*, *Proc. Natl. Acad. Sci. USA*, 87, 6547-6549, 1990).

At present,  $\alpha$ -interferon is widely used for treating HCV patients. However, only about half of the patients respond to  $\alpha$ -interferon, and about half of the responders suffer from a recurrence of the virus (Hino *et al.*, *C. J. Med. Virol.*, 42, 299-305, 1994; Tsubota *et al.*, *Hepatology*, 19, 1088-1094, 1994). Development  
5 of other anti-HCV drugs is, therefore, necessary.

In order to develop the anti-HCV drugs, it is essential to set up HCV cultivation system to test the efficacy of the drugs. However, in vitro cultivation systems available to date are inefficient and technically difficult to manipulate (Yoo  
10 *et al.*, *J. Virol.*, 69, 32-38, 1995; Beach *et al.*, *Viral Hepatitis Research Foundation of Japan*, 286, 67, 1993; Shimizu *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 5477-5481, 1992). Furthermore, HCV titer is very low even in sera of hepatitis patients due to its nature of low yield. Accordingly, it is very difficult to screen or test for efficacy of antiviral drugs using HCV cultivation. Therefore, the surrogate virus  
15 will be very useful for studying HCV genes in virus replication and testing antiviral efficacy of drugs affecting HCV-encoded enzymes. Namely, the development of the surrogate virus for anti-HCV drugs is urgent.

### **SUMMARY OF THE INVENTION**

Accordingly, the present invention is intended to overcome the above-mentioned disadvantage in the development of anti-HCV drugs and provide  
20 proper surrogate viruses for HCV suitable for the investigation of antiviral efficacy of drugs and proliferable in the HCV cultivation system.

An embodiment of the present invention is a recombinant gene coding hepatitis C surrogate virus comprising a virus gene coding picornavirus, a

protease gene coding hepatitis C virus protease NS3 which is in an open reading frame (ORF) of said picornavirus, and a target gene coding target sites of said hepatitis C virus protease NS3 which is in said open reading frame of said picornavirus.

5           Among the picornaviruses, the poliovirus is preferred.

          The target site is preferred to be selected from the group consisting of NS5A/5B, NS4A/4B and NS4B/5A.

          The hepatitis C surrogate virus protease NS3 is preferred to cleave the peptide bond between cysteine of P1 and glycine of P1' as a target site.

10           In another aspect, the present invention provides a screening method of anti hepatitis C virus drugs comprising the step of detecting a material which inhibits surrogate virus proliferation expressed by the recombinant gene for coding hepatitis C surrogate virus.

          In another aspect, the present invention provides a measuring method of  
15   anti-hepatitis C virus drugs activity comprising the steps of proliferating the surrogate virus expressed by the recombinant gene coding hepatitis C surrogate virus in the presence of anti-hepatitis C virus agents, and measuring the proliferating ability of the hepatitis C surrogate virus.

          The present invention, in another aspect, provides a hepatitis C surrogate  
20   virus comprising the recombinant gene for coding hepatitis C surrogate virus.

          The poliovirus's versatility is used in developing surrogate virus suitable for investigation of HCV protease activity.

HCV belongs to the family *Flaviviridae* whose members are enveloped viruses having a positive-sense RNA genome (Francki *et al.*, *Arch. Virol* 2(Sullo.), 223, 1991; Inchauspe *et al.*, *Proc. Natl. Acad. Sci.*, 88, 10292-10296, 1991; Miller *et al.*, *Proc. Natl. Acad. Sci.*, 87, 2957-2061, 1990; Takamizawa *et al.*, *J. Virol.* 65, 1105-1113, 1991). The RNA encodes a polyprotein (~3010 amino acids) with the following gene order: 5'-C'E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3'. During and/or after synthesis, the polyprotein is processed into functional proteins by host- and virus-encoded proteases. Core protein (C) and envelope proteins (E1 and E2) are believed to compose the structural elements of the virion particle.

The balance of the processed proteins is believed to function in replication processes of the virus inside the host cells. The signal peptidase on the endoplasmic reticulum is responsible for the generation of the N-terminus of E1, E2, and possibly NS2. It has been suggested that the NS2/NS3 junction is cleaved by an HCV-encoded metalloprotease, which resides between the C-terminal portion of NS2 and the N-terminal region of NS3. The cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B sites depends on the activity of the serine protease NS3 (Bortenschlager *et al.*, *J. Virol.* 68, 5045-5055, 1993; Eckart *et al.*, *Biochem. Biophys. Res. Commun.* 192, 399-406, 1993; Grakoui *et al.*, *J. Virol.* 67, 2832-2843, 1993; Han *et al.*, *J. Gen. Virol.* 76, 985-993, 1995; Hijikata *et al.*, *J. Virol.* 67, 4665-4675, 1993; Tomei *et al.*, *J. Virol.* 67, 4017-4026, 1993).

HCV protease NS3 is a good putative therapeutic target molecule for the development of anti-HCV drugs, since its activity is considered essential for viral proliferation. Protease activity of NS3 resides in the N-terminal part of NS3

(Bartenschlager *et al.*, *J. Virol.* 67, 3835-3844, 1994; Han *et al.*, *J. Gen Virol.* 76, 985-993, 1995; Lin *et al.*, *J. Virol.* 68, 8147-8157, 1994), and NS4A enhances the protease activity of NS3 (Bartenschlager *et al.*, *J. Virol.* 68, 3835-3844, 1994; Failla *et al.*, *J. Virol.* 68, 3753-3760, 1994; Hahm *et al.*, *J. Virol.* 69, 2534-2539, 1995; Lin *et al.*, *J. Virol.* 69, 4373-4380, 1995; Tanji *et al.*, *J. Virol.* 69, 1575-1581, 1995). Characterization of the NS3 protease using known protease inhibitors has revealed that NS3 is a chymotrypsin-like protease (Hahm *et al.*, *J. Virol.* 69, 2534-2539, 1995).

The poliovirus is the prototype of picornaviruses. The polioviral genome is composed of a positive-sense RNA molecule that encodes a single open reading frame (ORF) (Kitamura *et al.*, *Nature* (London) 291, 547-553, 1981; Racaniello *et al.*, *Proc. Natl. Acad. Sci. USA* 78, 4887-4891, 1981). Upon infection, the genomic RNA is translated into a large precursor polyprotein via the internal ribosomal entry site (IRES) in the 5'-nontranslated region of the RNA (Jang *et al.*, *J. Virol.* 62, 2636-2643, 1988; Pelletier *et al.*, *Nature* (London) 334, 320-325, 1988; Pelletier *et al.*, *J. Virol.* 63, 441-444, 1989). The polyprotein, in turn, is processed into the mature viral structural and nonstructural proteins by the 2A, 3C, and 3CD proteases (Harris *et al.*, *Semin. Virol.* 1, 323-333, 1991; Lawson and Semler., In "Current Topics in Microbiology and Immunology" (V.R. Racaniello, Ed.), 161, 49-87, 1990). Poliovirus is one of the best studied viruses and is relatively easy to manipulate and cultivate. Production of infectious poliovirus from cDNA clones has been practiced for a decade to study functions of poliovirus proteins (Molla *et al.*, *Science* 254, 1647-1651, 1991; Van der Werf *et al.*, *Proc. Natl. Acad. Sci.*

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USA 83, 2330-2334, 1986) or for use of the poliovirus as a vector in delivering foreign genes into host cells (Alexander *et al.*, *Proc. Natl. Acad. Sci. USA* 91, 1406-1410, 1994; Andino *et al.*, *Science* 265, 1448-1451, 1994; Lu *et al.*, *J. Virol.* 69, 4797-4806, 1995)

5           Inventors generated a hybrid poliovirus which requires the activity of HCV protease NS3 for its proliferation, on the basis of the characteristics of HCV and poliovirus. The hybrid virus is composed of poliovirus and HCV. The proteins necessary for the proliferation and infection into other cells are produced by poliovirus gene for the hybrid virus. The poliovirus gene is preceded by HCV  
10           protease and the sites cleaved by the protease to construct this hybrid virus which requires HCV protease NS3 activity for viability. This virus is composed, sequentially, HCV NS3 protease domain plus its target site, and open reading frame of poliovirus which is necessary for virus replication and virus particle formation. The activity of the enzyme NS3 protease is necessary to survive the  
15           hybrid virus.

          The hybrid virus is proliferable in the cultivation system to settle the conventional problem which HCV cannot be proliferated in vitro cultivation systems. Therefore, the hybrid virus enables to develop anti-HCV drugs based on protease inhibitor easily and precisely.

#### 20           DETAILED DESCRIPTION OF THE DRAWINGS

          The present invention will be described with reference to the accompanying drawings, in which:



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FIG. 1 is schematic diagram of the genomic organization of poliovirus type 1 (Mahoney), NS3  $\Delta$  C-PV1, and NS3  $\Delta$  C'-PV1.

FIG. 2 is a photograph of plaque phenotypes of PV1 (M) and vNS3  $\Delta$  C-PV1.

5 FIG. 3 is a photograph of RT-PCT analysis of vNS3  $\Delta$  C-PV1 RNA.

Lane 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as template of pNS3  $\Delta$  C-PV1, (+) sense RNA and (-) sense RNA, respectively.

FIGs. 4A, 4B and 4C are photographs of proteolytic processing patterns of PV1, NS3  $\Delta$  C-PV1, and NS3  $\Delta$  C'-PV1 polyproteins.

10 FIG. 5 is one-step graph curves of PV1 (M) and vNS3  $\Delta$  C-PV1. HeLa cells were infected with either PV1 or vNS3  $\Delta$  C-PV1 at a multiplicity of infection of 10.

FIG. 6A to FIG. 6E are shown the genetic sequence of pNS3  $\Delta$  C-PV1 according to an embodiment of the present invention.

15 **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

In the following detailed description, only the preferred embodiment of the invention has been shown and described, simply by way of illustration of the best mode contemplated by the inventor(s) of carrying out the invention. As will be realized, the invention is capable of modification in various obvious respects, all without departing from the invention. Accordingly, the drawing and description are to be regarded as illustrative in nature, and not as restrictive.

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## EXAMPLE

### Material and Methods

#### Construction of plasmids

DNA manipulations were carried out by standard methods (Sambrook *et al.*, Molecular cloning: a laboratory manual (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). The construction of the plasmids pTHE  $\Delta$  1219-1634 and pPNENPO are described elsewhere (Han *et al.*, *J. Gen. Virol.*, 76, 985-993, 1995; Alexander *et al.*, *Proc. Natl. Acad. Sci., USA*, 91, 1406-1410, 1994). pUC19 was cleaved with XbaI, treated with Klenow, and digested with PstI. The resulting 2.6 kb fragment was then ligated to the 1.3 kb fragment of pTHE $\Delta$ 1219-1634 treated with NcoI, Klenow, and PstI in succession. The result was the construct pUC19-NS3  $\Delta$  C-4  $\Delta$  C. pPNENPO and pUC19-NS3  $\Delta$  C-4  $\Delta$  C were digested with EcoRI or KpnI, respectively, and treated with Klenow enzyme. The linearized DNAs were ligated to result in the construct pPNENPO-NS3  $\Delta$  C-4  $\Delta$  C.

To construct pNS3  $\Delta$  C-4A-PV1, a polymerase chain reaction (PCR) was performed using the following four oligonucleotides:

Primer 1: 5'-TGATATCGAATTCCGG-3';

Primer 2: 5'-ATATGAGCTCCGCACTCTTCCATCTC-3';

Primer 3: 5'-ATATGAGCTCAGGTTTCATCACAGAAAG-3';

Primer 4: 5'-CTGTGCTAGCGCTTTTGG-3'.

The primers 1 plus 2 and pPNENPO-NS3  $\Delta$  C-4  $\Delta$  C were used to

amplify the HCV protease NS3  $\Delta$  C-4A region (PCR product 1). The PCR product 1 contains a new SacI site right after the NS4A-coding sequence. The primers 3 plus 4 and pPNENPO were used to amplify polioviral cDNA (746-2479) with a new SacI site at the beginning of the poliovirus ORF (PCR product 2).  
5 EcoRI and NheI digested pPNENPO, EcoRI and SacI digested PCR product 1, and SacI and NheI digested PCR product 2 were ligated together to generate plasmid pNS3  $\Delta$  C-4A-PV1.

To construct pNS3  $\Delta$  C-PV1, an oligonucleotide (5'-AGCTCCGCAGCAGACGACGTCCTCACTGGCTTCCTCTTGCGCGCCTCCTCCT  
10 CCGGTTGAGTTATCTGTGAAGAC-3') encoding the C-terminal end of NS5A (P1-P10) and primer 1 were used to amplify the HCV region encoding amino acids 1016-1215. The PCR product was phosphorylated with polynucleotide kinase and then digested with EcoRI. pNS3  $\Delta$  C-4A-PV1 was cleaved with SacI, treated with T4 DNA polymerase, and then digested with EcoRI. The larger  
15 fragment of pNS3  $\Delta$  C-4A-PV1 was ligated to the PCR product to generate pNS3  $\Delta$  C-PV1. The junction sequences were confirmed by sequencing.

Site-directed mutagenesis of the serine in the catalytic triad of the HCV protease (Ser1165Ala).

Site-directed mutagenesis was carried out by the Kunkel method as  
20 previously described (Kunkel et al., *Methods Enzymol.*, 154, 367, 1987). pSK1016-1650 was generated by ligation of the DNA fragments isolated from PvuII plus Sall-digested pTHE1016-1846 and SmaI plus Sall-digested pBluescript

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SK(+). E. coli strain RZ 1032 (ung<sup>r</sup> dut<sup>r</sup>) was transformed with pSK1016-1650, and ssDNA was prepared from the transformant. An oligonucleotide, 5'-CTTGAAGGGCTCCGCG GGTGG-3', designed to change the serine residue in the catalytic triad of HCV protease to alanine [Ser 1165(TCG) to Ala(GCG)] was used for site directed mutagenesis. The oligonucleotide was phosphorylated with T4 polynucleotide kinase (Boehringer Mannheim) and annealed to the single stranded pSK1016-1650 DNA, extended with sequenase (USB), and ligated with T4 DNA ligase (Poscochem.) to result in covalently closed, circular, dsDNA. The ligated dsDNA was introduced into E. coli XL1-blue. The base change was confirmed by the presence of a newly generated SacII site and by sequencing. pSK1016-1650 (Ser1165Ala) was used to construct pNS3  $\Delta$  C'-PV1 by replacing the XmaI fragment of pNS3  $\Delta$  C-PV1 with the same fragment of pSK1016-1650 (Ser1165Ala).

#### In vitro transcription and translation

Plasmid DNAs were purified following the polyethylene glycol precipitation method. They were then linearized with appropriate restriction enzymes downstream of the translation termination codon. The linearized DNAs were extracted with phenol/chloroform and ethanol-precipitated. RNAs were transcribed from the purified DNAs with T7 RNA polymerase (Boehringer Mannheim) as described by the manufacturer. These RNA transcripts were translated in a rabbit reticulocyte lysate system (RRL) supplemented with 25% HeLa cell lysate in the presence of [<sup>35</sup>S] methionine. The *in vitro* translation reactions were carried out at 30 °C for 8 hours. The [<sup>35</sup>S]-labeled proteins were

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analyzed on 15% SDS-PAGE using the buffer system described by Nicklin et al. (1987). The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. Gels were dried and exposed to Kodak XAR-5 film or Agfa Curix RP1 film.

5        RNA transfection

An electroporation protocol was used to transfect HeLa cell monolayers with the viral RNAs prepared *in vitro*. Cells grown to 80% confluency were harvested. After two washes, the cells were resuspended in the medium at a concentration of  $5 \times 10^6$  cells/ml. The cell suspension (1 ml) was mixed with 3  
10     $\mu$ g of RNA and placed in a 0.4 cm Gene Pulser cuvette (BIO-RAD). The cuvette was electrically pulsed at 360 volt and 960  $\mu$ F using the Gene Pulser transfection apparatus (BIO-RAD). Then the cells were diluted with 9 ml of medium containing 10 % bovine calf serum, 5 % equine serum, and 5 % fetal calf serum.

Plaque assay and one-step growth curve

15        Titers of virus stocks were measured by plaque assay on HeLa cell monolayers as follows. Cells were inoculated with virus, left for 30 minutes at room temperature, and then overlaid with Eagle medium containing 0.9 % Noble agar and 5 % fetal calf serum. Viral plaques were visualized with 0.5 % crystal violet solution 60 hours postinfection.

20        To measure one-step growth kinetics, virus was loaded onto 3.5 cm HeLa cell plates at a multiplicity of infection of 10 per cell and incubated at room temperature for 30 minutes to allow virus binding. Unbound particles were

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removed by two rounds of PBS washing. The infected cells were cultured at 37 °C in 2 ml of Eagle medium containing 10 % bovine calf serum and harvested at the time points indicated in FIG. 5. The infectivity of the cell lysate at each time point was titrated by plaque assay.

5        Reverse transcription PCR (RT-PCR)

Total RNA from virus-infected cells was isolated 4 hours postinfection as described by Lu et al. (1995). cDNAs were synthesized using M-MuLV reverse transcriptase and oligonucleotide primers corresponding to poliovirus nucleotides 556-570 and 789-806 (primer 5: 5'-GTGTTTCCTTTTATT-3'; primer 6: 5'-GTGTTTCCTTTTATT-3'). Primer 5 and primer 6 were used in synthesizing cDNAs from negative- and positive-sense RNA, respectively. Both of the primers were used in the PCR reaction that followed. The PCR products were analyzed on a 1.3 % agarose gel.

10        Labeling of newly synthesized proteins in virus-infected cells

15        HeLa cells were infected with poliovirus 1 (Mahoney: PV1) and vNS3  $\Delta$  C-PV1 at a multiplicity of infection of 30 PFU per cell. The cells were incubated in a medium lacking methionine for 10 minutes 3 hours after infection. Subsequently, 100  $\mu$  Ci of [ $^{35}$ S] methionine was added to the medium and the incubation continued for 40 minutes. The cells were then collected and lysed.

20        The lysate was analyzed on 15 % SDS-PAGE.

Immunoblot analysis

Virus-infected cells were lysed 4 hours postinfection. Total protein (13

$\mu$ g) was resolved on a 15 % SDS-PAGE gel and transferred onto a nitrocellulose membrane (Amersham). The blot was incubated overnight at 4 °C in blocking solution [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 % Tween 20, and 5 % powdered skim milk] to block nonspecific binding. The primary antibody (polyclonal antibody against HCV protease NS3) was added to the blocking solution for 3 hours. The antibody was a generous gift from Dr. R. Bartenschlager, Institute of Virology, Mainz, Germany. A horseradish peroxidase-linked anti-rabbit IgG in a 1:10,000 dilution was used as the secondary antibody. Membrane-bound antibodies were detected by enhanced chemiluminescence reagents (ECL; Amersham).

Generation of a novel poliovirus containing HCV protease NS3 and its target site

In order to develop an evaluation system for anti-HCV drugs targeting protease NS3, a recombinant poliovirus that requires the activity of HCV protease for its proliferation, was constructed. A schematic diagram of the genomic structure of this virus is depicted in FIG. 1. The hatched and open boxes represent HCV protease NS3  $\Delta$  C and poliovirus polyprotein, respectively. The amino acid sequence at the junction of HCV protease NS3 and poliovirus P1 is indicated by single letter amino acid codes. The target site of HCV protease is marked by an arrowhead. The mutated amino acid (S1165A) in NS3  $\Delta$  C'-PV1 is indicated by an asterisk.

As shown in FIG. 1, in the case of polyprotein NS3  $\Delta$  C-PV1, the protease

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domain of HCV NS3 is fused to the last 10 amino acids of NS5A (EEASEDVVCC), which are recognized by protease NS3. These amino acids residues are preceded by four glycines, alanine, and glutamine serving as a hinge region between the protease domain and the target site. It was expected that the HCV protease NS3 would cleave the peptide bond between cysteine at the C-terminus of the additional polypeptide and glycine at the N-terminus of poliovirus P1, since HCV protease NS3 is known to cleave between the cysteine or threonine at the P1-residue and the small uncharged residues at the P1'-residue (Kolykhalov *et al.*, *J. Virol.* 68, 7525-7533, 1994; Komoda *et al.*, *J. Virol.*, 68, 7351-7357, 1994).

Upon transfection, the RNA transcript encoding NS3  $\Delta$  C-PV1 produced a virus designated vNS3  $\Delta$  C-PV1. On the other hand, the RNA transcript similar to NS3  $\Delta$  C-PV1 but containing a serine to alanine mutation at the catalytic triad of HCV protease NS3 (NS3  $\Delta$  C'-PV1) did not yield virus. This strongly suggests that HCV protease NS3 activity is essential for the viability of the hybrid virus vNS3  $\Delta$  C-PV1. The virus vNS3  $\Delta$  C-PV1 has a small plaque phenotype at 37 °C as shown in FIG. 2. (compare plaque sizes in panel PV1 with vNS3  $\Delta$  C-PV1) or at 30 °C (data not shown).

The virus vNS3  $\Delta$  C-PV1 is stable through at least five passages. The integrity of vNS3  $\Delta$  C-PV1 was confirmed by analyzing the viral RNAs in cells inoculated with a virus stock of the fourth passage. Lanes 1, 2 and 3 depict DNAs produced by PCR or RT-PCR using as templates plasmid pNS3  $\Delta$  C-PV1, (+) sense RNA, respectively. The inserted HCV sequence in the poliovirus



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genome was amplified by RT-PCT with primers annealing to the poliovirus 5' nontranslated region and the P1-coding region. The viral RNAs of the fifth passage retained the inserted HCV protease and NS5A sequences as shown in FIG. 3. Both positive and negative sense viral RNAs of the expected sizes were detected (FIG. 3, lanes 2 and 3, respectively). RT-PCR analysis of the genomic RNAs isolated from vNS3  $\Delta$  C-PV1 virion particles revealed the same RT-PCR products as the RNAs isolated from HeLa cells (data not shown). The junction sequence between HCV and poliovirus was confirmed by DNA-sequencing of the PCR products and the sequences of hepatitis C surrogate virus genome is presented in FIG. 6. This indicates that vNS3  $\Delta$  C-PV1 is stable at least for five passages.

Proper proteolytic processing of vNS3  $\Delta$  C-PV1 polyprotein requires HCV

NS3 protease activity.

Proteolytic processing patterns of polyproteins encoded by poliovirus, vNS3  $\Delta$  C-PV1, and NS3  $\Delta$  C'-PV1 RNA were analyzed by *in vitro* transcription and translation of pT7-PC1, pNS3  $\Delta$  C-PV1, and pNS3  $\Delta$  C'-PV1. Most of the polioviral proteins were detected as *in vitro* translation products of the PV1 RNA transcript (FIG. 4A, lane 1). Two additional protein bands of 123 kDa and 26 kDa were apparent among the translation products of NS3  $\Delta$  C-PV1 as compared to the PV1-translation product (FIG. 4A, lane 2). The 123 kDa and 26 kDa proteins are most likely NS3  $\Delta$  C-P1 fusion protein and the processed NS3  $\Delta$  C, respectively, since the cleavage of poliovirus at the P1/P2 junction occurs very

fast, even during the process of translation. Translation of vNS3  $\Delta$  C'-PV1 also yielded the 123 kDa protein. But the 26 kDa and the P1 protein were not detected among the translation products from this construct (FIG. 4A, lane 3). This indicates that proteolytic cleavage of the HCV/poliovirus junction does not occur in NS3  $\Delta$  C'-PV1, and that the cleavage of this junction is mediated by the protease activity of NS3. It also shows that the failure of virus production with the NS3  $\Delta$  C'-PV1 construct is most likely due to the lack of the P1 polypeptide which is a protein processed into viral coat protein (VP0, VP1, and VP3). In other words, HCV NS3 activity is essential for proliferation of the vNS3  $\Delta$  C-PV1. Interestingly, complete processing of the viral capsid proteins (VP0, VP1, and VP3) from the NS3  $\Delta$  C'-PV1 polyprotein was not detected. On the other hand, the processed capsid proteins were apparent along with the precursor NS3  $\Delta$  C-P1 (compare lane 2 with lane 3 in FIG. 4A). The blockage of proteolytic processing of the NS3  $\Delta$  C'-PV1 polyprotein may be due to the lack of myristoylation of the glycine residue at the N-terminus of P1 that has been shown to be important in the processing of P1 by poliovirus protease 3C-3D (Kräusslich *et al.*, *J. Virol.*, 64, 2433-2436, 1990).

The proteolytic processing was also investigated for PV1 and vNS3  $\Delta$  C-PV1 by labeling newly synthesized proteins with [<sup>35</sup>S] methionine in virus-infected cells. The labeling in vNS3  $\Delta$  C-PV1-infected cells showed four additional bands with apparent molecular weights of 123 kDa, 89 kDa, 63 kDa and 26 kDa as indicated by arrow heads (FIG. 4B, compare lane 2 with lane 3). These proteins

are most likely NS3  $\Delta$  C-P1, NS3  $\Delta$  C-VP0-VP3, NS3  $\Delta$  C-VP0, and NS3  $\Delta$  C, respectively (FIG. 4B). This assumption is supported by the Western blotting analysis using an antibody against HCV NS3 and HeLa cell extracts after infection with either wild type poliovirus or vNS3  $\Delta$  C-PV1 (FIG. 4C). Among the proteins

5 of vNS3  $\Delta$  C-PV1-infected cells, the anti-NS3 antibody recognized the 63 kDa and the 26 kDa polypeptides corresponding to vNS3  $\Delta$  C-VP0 and NS3  $\Delta$  C, respectively (FIG. 4C, lane.3). A longer exposure of the X-ray film also revealed the 123 kDa band and the 89 kDa band corresponding to NS3  $\Delta$  C-P1 and NS3  $\Delta$  C-VP0-VP3, respectively (data not shown). On the other hand, no bands were

10 detected in the Western analysis of cell extracts from wild type poliovirus- or mock-infected cells (FIG. 4C, lanes 1 and 2). The results indicate that proteolytic processing of the artificial polyprotein occurs as expected, even though the HCV/poliovirus junction cleavage happens slower than the P1/P2 junction cleavage. The relative intensities of the NS3-related bands varied depending on

15 the methodologies used to detect the polypeptides. For instance, a strong band of NS3  $\Delta$  C-P1 was detected by *in vitro* and *in vivo* labeling, but only a weak band of NS3  $\Delta$  C-P1 was detected by Western blot analysis. The [<sup>35</sup>S] methionine labeling method reveals only newly synthesized polypeptides and larger proteins, containing more methionine residues, give stronger signals. Western blot

20 analysis, on the other hand, reveals all of the proteins interacting with the primary antibody, and the intensity of the signal reflects the molarities of the proteins rather than their size. Therefore, [<sup>35</sup>S]methionine labeling methods are expected

to give relatively stronger signals for precursor forms than Western blot analysis methods.

One-step growth kinetics of chimeric virus vNS3  $\Delta$  C-PV1

FIG. 5 was one step growth curve for vNS3  $\Delta$  C-PV1. While maximum  
5 production of wild type poliovirus was reached 4 hours postinfection (FIG. 5, open squares), that of vNS3  $\Delta$  C-PV1 was delayed by about 2 hours (FIG 5, closed circles). In FIG. 5, the titer of vNS3  $\Delta$  C-PV1 at the peak was about 1<sup>1</sup>.5 orders of magnitude lower than that of wild type poliovirus, as viral titers at 9 hours postinfection was compared. The small plaque phenotype of vNS3  $\Delta$  C-PV1  
10 may thus be a result of delayed virion particle formation and smaller burst size.

The HCV protease NS3 activity is necessary for noble poliovirus according to the present invention to survive. This virus is useful for testing the efficacy of anti-virus drugs which targets protease NS3.

**WHAT IS CLAIMED IS:**

1. A recombinant gene coding hepatitis C surrogate virus

comprising:

a virus gene coding picornavirus;

5 a protease gene coding hepatitis C virus protease NS3 which is in an open reading frame (ORF) of said picornavirus; and

a target gene coding target sites of said hepatitis C virus protease NS3 which is in said open reading frame of said picornavirus.

2. The recombinant gene coding hepatitis C surrogate virus of claim

10 1, wherein said picornavirus is poliovirus.

3. The recombinant gene coding hepatitis C surrogate virus of claim

1, wherein said target site is selected from the group consisting of NS5A/5B, NS4A/4B and NS4B/5A.

4. The recombinant gene coding hepatitis C surrogate virus of claim

15 1, wherein said hepatitis C surrogate virus protease NS3 cleaves the peptide bond between cysteine of P1 and glycine of P1' as a target site.

5. A screening method of anti hepatitis C virus drugs comprising the step of:

detecting a material which inhibits surrogate virus proliferation expressed

20 by said recombinant gene for coding hepatitis C surrogate virus of claim 1.

6. A measuring method of anti-hepatitis C virus drugs activity

comprising the steps of:

proliferating the surrogate virus expressed by said recombinant gene  
coding hepatitis C surrogate virus of claim 1 in the presence of anti-hepatitis C  
virus drugs; and

measuring the proliferating ability of said hepatitis C surrogate virus.

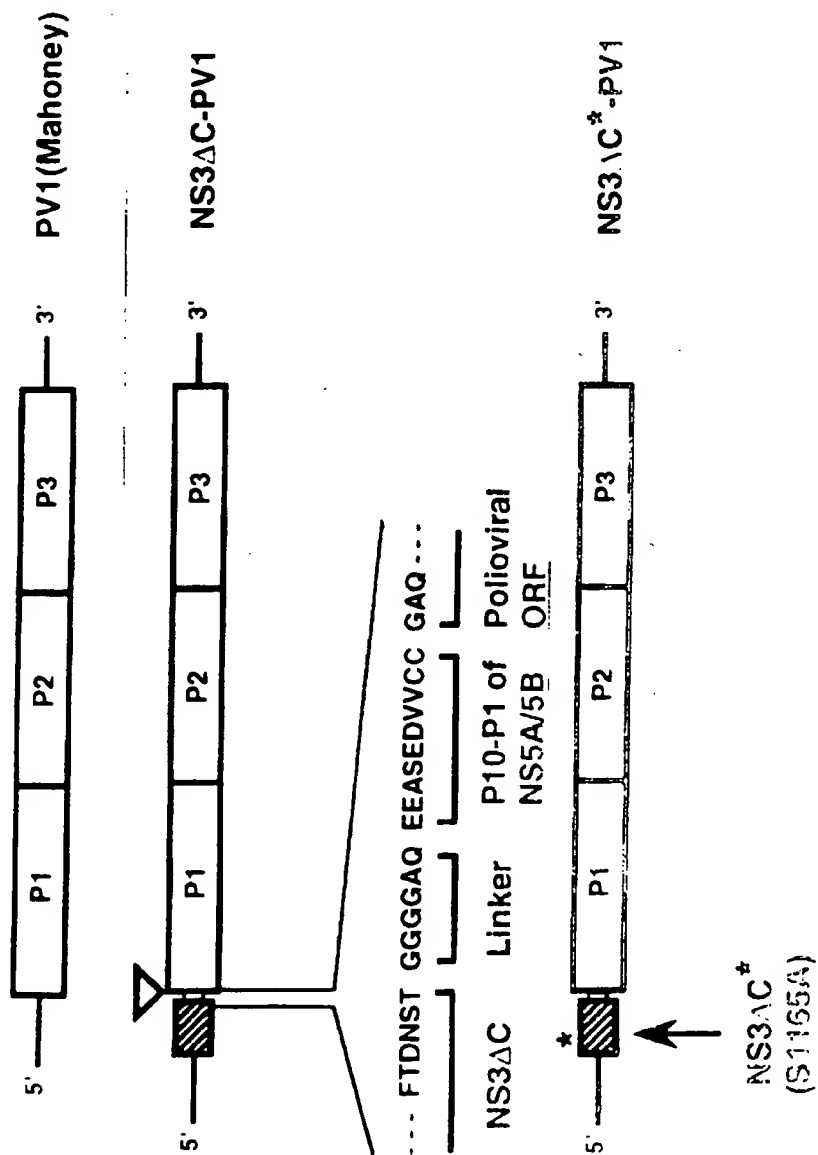
5

7. A hepatitis C surrogate virus comprising:

a recombinant gene for coding hepatitis C surrogate virus of claim 1.

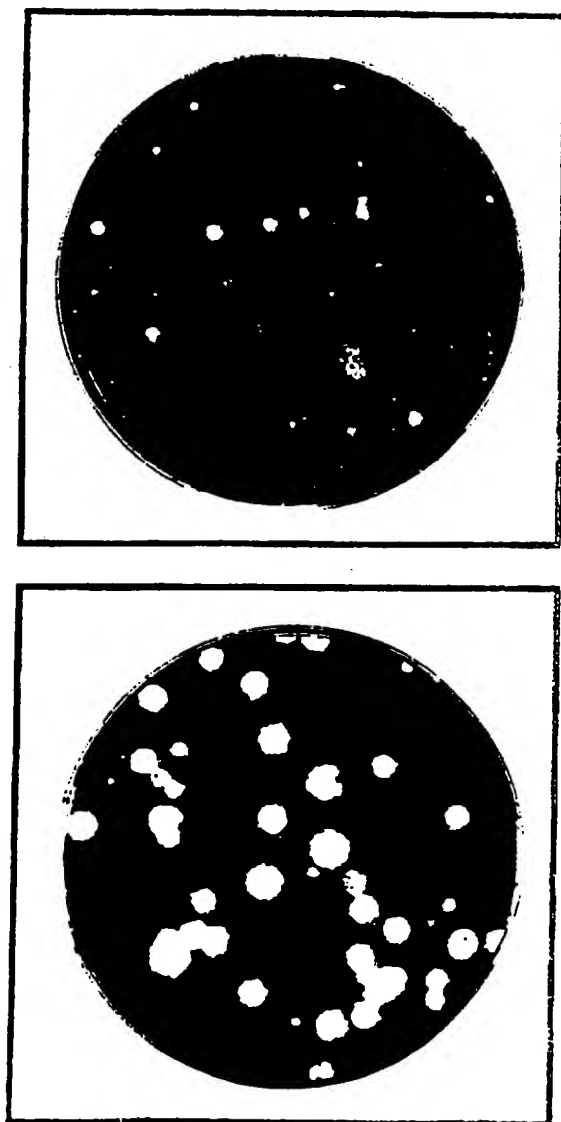
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FIG. 1



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FIG. 2



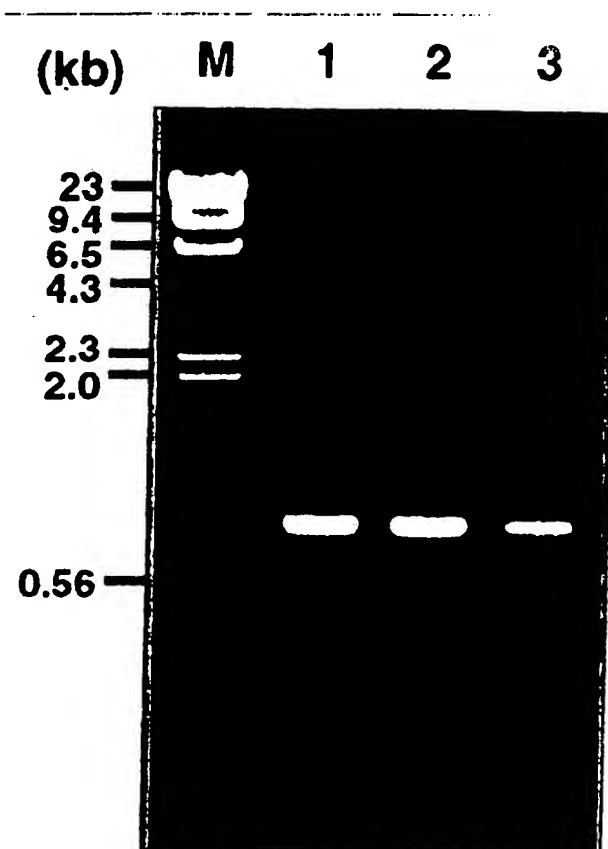
PV1

vNS3ΔC-PV1



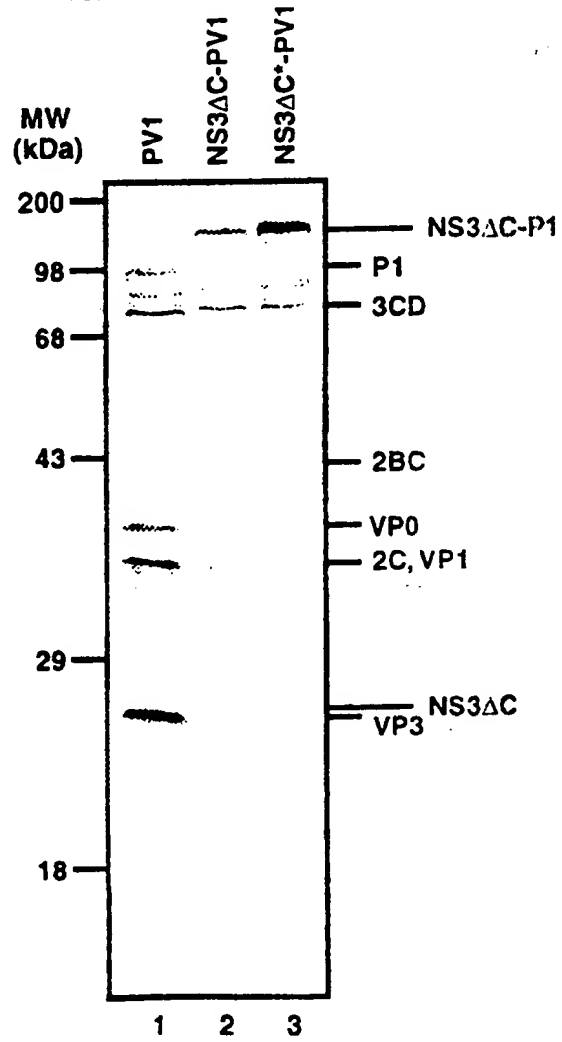
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## FIG. 3



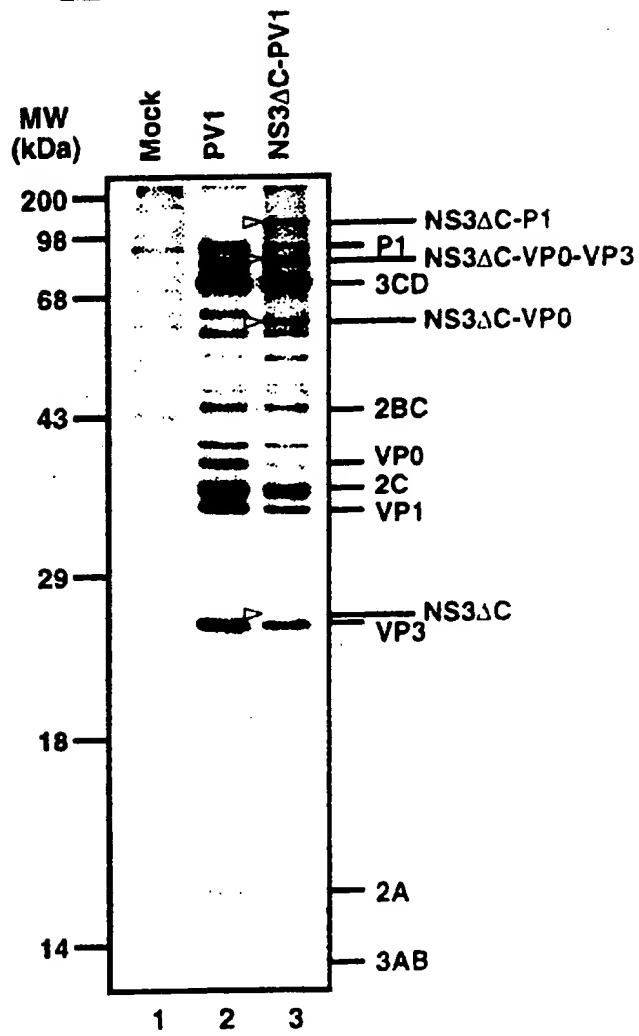
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# FIG. 4A



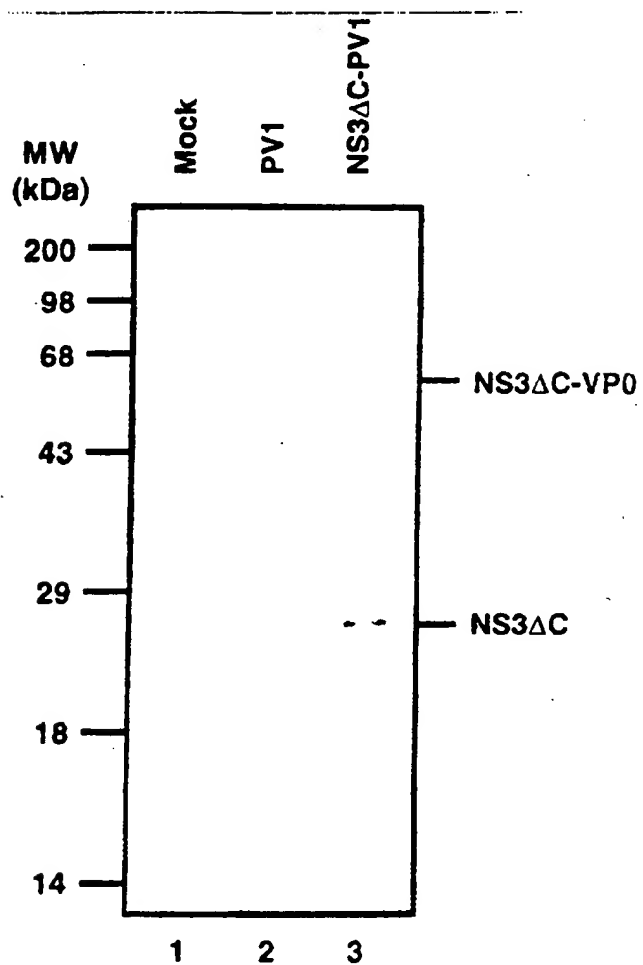
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# FIG. 4B



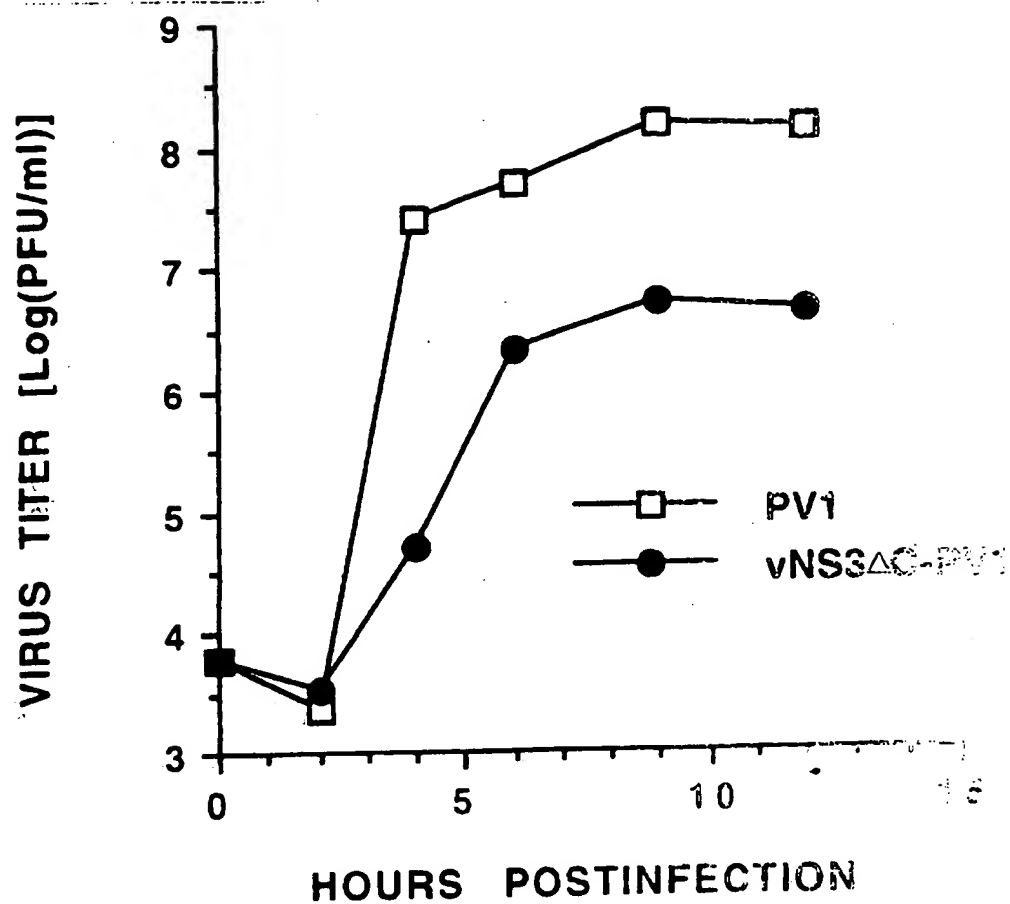
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## FIG. 4C



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FIG. 5



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## FIG. 6A

TTAAAACAGCTCTGGGGTTGTACCCACCCAGAGGCCACGTGGCGGCTAGTACTCCGGT  
ATTGCGGTACCCCTTGTACGC  
CTGTTTTATACTCCCTTCCCGTAACTTAGACGCACAAAACCAAGTTCAATAGAAGGGGG  
TACAAACCAGTACCACCACGA  
ACAAGCACTTCTGTTTCCCGGTGATGTCGTATAGACTGCTTGCCTGGTTGAAAGCGACG  
GATCCGTTATCCGCTTATGT  
ACTTCGAGAAGCCAGTACCACCTCGGAATCTTCGATGCGTTGCGCTCAGCACTCAACCC  
CAGAGTGTAGCTTAGGCTGA  
TGAGTCTGGACATCCCTCACCGGTGACGGTGGTCCAGGCTGCGTTGGCGGCTACCTATG  
GCTAACGCCATGGGACGCTA  
GTTGTGAACAAGGTGTGAAGAGCCTATTGAGCTACATAAGAATCCTCCGGCCCCCTGAAT  
GCGGCTAATCCCAACCTCGGA  
GCAGGTGGTCAAAAACAGTGATTGGCCTGTCGTAACGCGCAAGTCCGTGGCGGAACCGA  
CTACTTTGGGTGTCCGTGTT  
TCCTTTTATTTTATTGTGGCTGCTTATGGTGACAATCACAGATTGTTATCATAAAGCGA  
AGACGGTATAGCTTGATATCG  
AATTCCGGGGATCCTCTAGCATGGCCAGTCATCATCATCATCATCATGGAATTGTGAC  
AATCCCCGGCCGATAGCCTT  
GAAGGGCAGGGGTGGCAACTCCCCGCTCCCATCACGGCCTACTCCCAACAGACGCGGGGGC  
TACTTGGTTGCATCATCAC  
TAGCCTCACAGGCCGGGACAAGAACCAGTCGAGGGGGAAGTTCAAAGTGGTTTCCACCGC  
AACACAATCTTTCTGGCGA  
CCTGCGTCAATGGCGCTTGGACTGTCTTCCATGGTGCCGGCTCAAAGACCCTAGCCGGCCC  
AAAGGGGCCAATTACCAA  
ATGTACACCAATGTAGACCTGGACCTCGTCGGCTGGCAGGCACCCCCCGGGTCGCGTCCCC  
TGACACCATGCACCTGCGG  
CAGCTCAGACCTTTACTTGGTACGAGACATGCTGATGTATTCCGGTGCGCCGGCGGGG  
CGACAGTAGGGGGAGCCTAC  
CCTCTCCACAGACCACTCTCTACTTGAAGGGCTCCTCGGGTGGTCCACTGCTCTGCCCTTC  
GGGGCAGCTGTGTGGCATC  
TTTCGGGCTGCTGTATGCACCCGGGGGTTGCGAAGGCGGTGGACTTCATACCCGTTGAA  
TCTATGGAACTACTATGCG  
GTCTCCGCTCTTACAGATAACTCAACCGAGGAGGAGGCGCGCAAGAGGAAGCCAGTGA  
GGACGTGCTGCTGCTGCGGAG  
CTCAGGTTTCATCACAGAAAGTGGGCGCATGAAAACCTCAAATAGAGCGTATGGTGGT  
TCTACCATTAAATTACACCACC  
ATTAATTATTATAGAGATTACGCTAGTAACGCGGCTTCGAAACAGGACTTCTCTCAAGA  
CCCTTCCAAGTTACCGAGCC  
CATCAAGGATGTCTGATAAAAACAGCCCCAATGCTAAACTCGCCAAACATAGAGGCTT  
GCGGGTATAGCGATAGAGTAC  
TGCAATTAACACTGGGAACTCCACTATAACCACACAGGAGGCGGCTAATTCAGTAGTC  
GCTTATGGGCGTTGGCCTGAA  
TATCTGAGGGACAGCGAAGCCAATCCAGTGGACAGCCGACAGAACAGACGTCGCTGCA  
TGCAGGTTTTATACGCTAGA  
CACCGTGTCTTGGACGAAAGAGTCGCGAGGGTGGTGGTGGAAAGTTGCCTGATGCACTGAG  
GGACATGGGACTCTTTGGC  
AAAATATGTACTACCACTACCTAGGTAGGTCCGGGTACACCGTGCATGTACAGTGTAAAC  
GCCTCCAAATTCCACCAGGGG  
GCACTAGGGGTATTGCGCCTACACAGAGATGTGTCTGGCCGGGGATAGCAACACCCTACC  
ATGCACACCAGCTATCAAAA  
TGCCAATCCTGGCGAGAAAGGAGGCACTTTACGGGTACGTTCACTCCTGACAACAACCA  
GACATCACCTGCCCCGAGGT  
TCTGCCCGGTGGATTACCTCCTTGGAAATGGCACGTTGTTGGGGAATGCCTTTGTGTTCC  
CGCACCAGATAATAAACCTA  
CGGACCAACAACCTGTGCTACACTGGTACTCCCTTACGTGAACTCCCTCTCGATAGATAGT  
ATGGTAAAGCACATAATTG  
GGGAATTGCAATATTACCATTTGGCCCCATTAAATTTTGCTAGTGAGTCCTCCCCAGAGA  
TTCCAATCACCTTGACCATAG

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FIG. 6B

CCCCATGTGCTGTGAGTTCAATGGATTAAGAAACATCACCCCTGCCACGCTTACAGGGCC  
TGCCGGTCATGAACACCCCT  
GGTAGCAATCAATATCTTACTGCAGACAACTTCCAGTCACCGTGTGCGCTGCCCTGAATT  
TGATGTGACCCACCTATTGA  
CATACCCCGGTGAAAGTAAAGAACATGATGGAAATTGGCAGAAATCGACACCATGATTCCCT  
TTGACTTAAGTGCCACAAAA  
AGAACACCATGGAAATGTATAGGGTTTCGGTTAAGTGACAAACCACATACAGACGATCCC  
ATACTCTGCCCTGTCACTCTCT  
CCAGCTTCAGATCCTAGGTTGTACATACTATGCTTGGAGAAATCCTAAATTACTACAC  
ACACTGGGCAGGATCCCTGAA  
GTTACCGTTTCTGTTCTGTGGATCCATGATGGCAACTGGCAAACTGTTGGTGTATACG  
CGCTCCTGGAGCCGACCCAC  
CAAAGAAAGCGTAAGGAGGCGATGTTGGGAACACATGTGATCTGGGACATAGGACTGCAG  
TCCTCATGTACTATGGTAGTG  
CCATGGATTAGCAACACCAACGATATCGGCCAAACCATAGATGATAGTTTCACCGAAGGCGG  
ATACATCAGCGTCTTCTACCA  
AACTAGAAATAGTCGTCCTCTTTTCGACACCCAGAGAGATGGACATCCTTGGTTTTGTGT  
CAGCGTGTAATGACTTCAGCG  
TGCGCTTGTTCGAGATACCAACATATAGAGCAAAAAGCGCTAGCACAGGGGTTAGGT  
CAGATGCTTGAAAGCATGATT  
GACAACACAGTCCGTGAAACGGTGGGGGCGGCAACATCTAGAGACGCTCTCCAAACACT  
GAAGCCAGTGGACCAACACA  
CTCCAAGGAAATTCGGGCACTCACCGCAGTGGAAACTGGGGCCACAAATCCACTAGTCCC  
TTCTGATACAGTGCAAAACA  
GACATGTTGTACAACATAGGTCAAGGTCAGAGTCTAGCATAGAGTCTTTCTTCGCGCGG  
GGTGATGCGGTGACCAATTATG  
ACCGTGGATAACCCAGCTTCCACCACGAATAAGGATAAGCTTTTTGTCAGTGTGGAAGAT  
CACTTATAAAGATACTGTCCA  
GTTACGGAAGAAATTGGAGTCTTTCACCTATTCTAGATTTGATATGGAACCTTACCTTTG  
TGTTACTGCAAAATTTCACTG  
AGACTAACAATGGCCATGCAATTAATCAAGTGTACCAAAATTATGTACGTACCACCAAGG  
GCTCCAGTGCCGAAAAATGG  
GACGACTACATGSCAAACCTCATCAATCCATCAATCTTTTACACCTACGGGACAGCT  
CCAGCCCGGATCTCGGTACC  
GTATGTTGGTATTTGGAACGCTATTTCACACTTTTACGACGGTTTTTCCAAAGTACCAC  
TGAAGGACCAAGTCGGCAGCAC  
TAGGTGACTCCCTTTATGGTGACGATCTCTAAATGACTTCGGTATTTTGGCTGTTAGA  
GTAGTCAATGATCACAACCCG  
ACCAAGGTCACCTCCAAATCAGAGTGTATCTAAAACCCAAACACATCAGAGTCTGGTG  
CCCGCGTCCACCGAGGGCAGT  
GGCGTACTACGGCCCTGGAGTGGATTACAAGGATGGTACGCTTACACCCCTCTCCACCAA  
GGATCTGACCACATATGGAT  
TCGGACACCAAAACAAAGCGGTGTACACTGCAGGTTACAAAATTTGCAACTACCACTTG  
GCCACTCAGGATGATTGCAA  
AACGCAGTGAACGTGATGTGGAGTAGAGACCTCTTAGTCACAGAATCAAGAGCCCAGGG  
CACCGATTCAATCGCAAGGTG  
CAATTGCAACGCAAGGGGTGTACTACTGCGAGTCTAGAAGGAAATACTACCCAGTATCCT  
TCGTTGGCCCAACGTTCCAGT  
ACATGGAGGGCTAATAACTATTACCCAGCTAGGTACCAGTCCCATATGCTCATTGGCCAT  
GGATTGCGATCTCCAGGGGAT  
TGTGGTGGCATACTCAGATGTACCACGGGGGTGATAGGGATCATTACTGCTGGTGGAGA  
AGGGTTGGTTGCATTTTCA  
CATTAGAGACTTGTATGCCTACGAAGAAAGGCCATGGAACAAGGCCTCACCAATTACA  
TAGAGTCACTTGGGCGCGCAT  
TTGGAAGTGGATTTACTCAGCAGATTAGCGACAAAATAACAGAGTTGACCAATATGGTG  
ACCAGTACCATCACTGAAAAAG  
CTACTTAAGAACTTGATCAAGATCATATCCTCACTAGTTATTATAACTAGGAACATATGA  
AGACACCACAACAGTGCTCGC

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## FIG. 6C

TACCCTGGCCCTTCTTGGGTGTGATGCTTCACCATGGCAGTGGCTTAGAAAGAAAGCATG  
CGATGTTCTGGAGATACCTT  
ATGTCATCAAGCAAGGTGACAGTTGGTTGAAGAAAGTTTACTGAAAGCATGCAACGCAGCT  
AAGGGCCTGGAGTGGGTGTCA  
AACAAAATCTCAAAATTCATTGATTGGCTCAAGGAGAAAATTATCCACAAGCTAGAGA  
TAAGTTGGAAATTGTAAACAA  
ACTTAGACAACCTAGAAATGCTGGAACCAAAATCTCAACTATACACCAATCATGCCCTA  
GTCAGGAACACCAAGGAATTC  
TATTCAATAATGTGATGTTATCCATCCAAGTCTAAGAGGTTTGCCCCCTCTTTACGCA  
GTGGAAGCCAAAAGAATACAG  
AAACTCGAGCATACTATTAACAACCTACATACAGTTCAAGAGCAAACACCGTATTGAACC  
AGTATGTTTGTCTAGTACATGG  
CAGCCCCGGAAACAGGTAAATCTGTAGCAACCAACCTGATTGCTAGAGCCATAGCTGAAA  
GAGAAACACGTCCACGTACT  
CGCTACCCCGGATCCATCACACTTCGACGGATACAAACAACAGGGAGTGGTGATTATGG  
ACGACCTGAATCAAAACCCA  
GATGGTGCCGACATGAAGCTGTTCTGTGATGTTATCAACAGTGGAGTTTATACCACC  
CATGGCATCCCTGGAGGAGAA  
AGGAATCCTGTTTACTTCAAATTACGTTCTAGCATCCACAACTCAAGCAGAATTTCCC  
CCCCCACTGTGGCACACAGTG  
ACGCGTTAGCCAGGCGCTTTGCGTTGACATGGACATTCAAGGTGATGAATGAGTATTCT  
AGAGATGGGAATTTGAACATG  
GCCATGGCTACTGAAATGTGTAAAGAACTGTCAACCAACCAGCAAACTTTAAGAGATGCTG  
TCCTTTAGTGTGTGGTAAGGC  
AATTCAATTAATGGACAAATCTTCAGAGTTAGATACAGTATTGACCAGATCACTACAA  
TGATTATCAATGAGAGAAACA  
GAAGATCCAAACATTGGCAATTGTATGGAGGCTTTGTTTCAAGGACCACTCCAGTATAAA  
GACTTGAAAATTGACATCAAG  
ACGAGTCCCCCTCCTGAATGTATCAATGACTTGCTCCAAGCAGTTGACTCCCAGGAGGTG  
AGAGATTACTGTGAGAAGAA  
GGGTGGATAGTTAACATCACCAGCCAGGTTCAAACAGAAAGGAACATCAACAGGGCAA  
TGACAATTTTACAAGCGGTGA  
CAACCTTCGCCGCAAGTGGCTGGAGTTGTCTATGTCTATATAAACTGTTTGCTGGACACC  
AGGAGCATACACTGTTTA  
CCAAACAAAAACCCAACTGCGCCACCATTGGACAGCAAGGTACAAGGACCAGGGTTC  
GATTACGCAAGTGGCTATGGC  
TAAAGAAACATTGTTACAGCAACTACTAGCAAGGGAGAGTTCACTATGTTAGGAGTCC  
ACGACAACGTGGCTATTTTAC  
CAACCCACGCTTCACCTGGTGAAAGCATTGTGATCGATGGCAAAGAGTGGAGATCTTG  
GATGCCAAAGCGCTCGAAGAT  
CAAGCAGGAACCAATCTTGAAATCACTATAATCACTCTAAAGAGAAATGAAAAGTTCAG  
AGACATTAGACCACATATACC  
TACTCAAACTCACTGAGACAAATGATGGAGTCTTGATCGTGAACACTAGCAAGTACCCCA  
ATATGTATGTTCTGTCTGGTG  
CTGTGACTGAACAGGGATATCTAAATCTCGGTGGGCGCCAAACTGCTCGTACTCTAATG  
TACAACCTTTCAACCAGAGCA  
GGACAGTGTGGTGGAGTCATCATGTACTGGGAAAGTCATCGGGATGCATGTTGGTGG  
GAACGGTTCACACGGGTTTGC  
AGCGGCCCTGAAGCGATCATACTTCACTCAGAGTCAAGGTGAAATCCAGTGGATGAGAC  
CTTCGAAGGAAGTGGGATATC  
CAATCATAAATGCCCGCTCCAAAACCAAGCTTGAACCCAGTGCTTTCCACTATGTGTTTG  
AAGGGGTGAAGGAACAGCA  
GTCCTCACTAAAAACGATCCCAAGGCTTAAGACAGACTTTGAGGAGGCAATTTTCTCCAA  
GTACGTGGGTAAACAAATTAC  
TGAAGTGGATGAGTACATGAAGAGGGCAGTAGACCACTATGCTGGCCAGCTCATGTAC  
TAGACATCAACACAGAACAA  
TGTGCTTGGAGGATGCCATGTATGGCACTGATGGTCTAGAAGCACTTGATTGTCCACC  
AGTGCTGGCTACCCTTATGTA



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FIG. 6D

GCAATGGGAAAAGAAGAAGAGAGACATCTTGAACAAACAAACCAGAGACACTAAGGAAA  
TGCAAAAACCTGCTCGACACATA  
TGGAAATCAACCTCCCACTGGTGACTTATGTAAAGGATGAACTTAGATCCAAAACAAAGG  
TTGAGCAGGGGAAAATCCAGAT  
TAATTGAAGCTTCTAGTTTGAATGACTCAGTGCCAAATGAGAATGGCTTTTGGGAACCTA  
TATGCTGCTTTTACAAAAAC  
CCAAGGAGTGATAACAGGTTTCAAGCAGTGGGGTGCGATCCAGATTGTGTTTGGAGCAAAAT  
TCCGGTATTGATGGAAGAGAA  
GCTGTTTGGCTTTTGAACACAGGGTATGATGCATCTCTCAGCCCTGCTTGGTTTCGAGGC  
ACTAAAGATGGTGCTTGAGA  
AAATCGGATTTCGGAGACAGAGTTGACTACATCGACTACCTAAACCACTCACACCACCTGT  
ACAAGAATAAAACATACTGT  
GTCAAGGGCGGTATGCCATCTGGCTGCTCAGGCACCTTCAATTTTAACTCAATGATTAA  
CAACTTGATTATCAGGACACT  
CTTACTGAAAACTACAAGGGCATAGATTTAGACCACCTAAAAATGATTGCCTATGGTG  
ATGATGTAATTGCTTCTTACC  
CCCATGAAGTTGACGCTAGTCTCCTAGCCCAATCAGGAAAAGACTATGGACTAACTATG  
ACTCCAGCTGACAAATCAGCT  
ACATTTGAAACAGTCACATGGGAGAATGTAACATTCTTGAAGAGATTCTTCAGGGCAGA  
CGAGAAATACCCATTTCTTAT  
TCATCCAGTAATGCCAATGAAGGAAATTCATGAATCAATTAGATGGACTAAAGATCCTA  
GGAACACTCAGGATCACGTTT  
GCTCTCTGTGCTTTTAGCTTGGCACAATGGCGAAGAAGAATATAACAAATTCCTAGCT  
AAAATCAGGAGTGTGCCAAT  
GGAAGAGCTTTATTGCTCCCAAGTACTCAACATTGTACCGCCGTTGGCTTGACTCATT  
TTAGTAACCCCTACCTCAGTCG  
AATTGGATTGGGTCTACTGTTGTAGGGGTAAATTTTTCTTTAATTCGGAGGAAAAAA  
AAAAAAAAAAAAAAAAAAAA  
AAACGGa  
atLAATTCTTGAAGACGAAAGGG  
CCTCGTGATACGCCTATTTTATAGGTTAATGTATGATAATAATGGTTTCTTAGACGT  
CAGGTGGCACTTTTCGGGAA  
ATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTC  
ATGAGACAATAACCCCTGATAA  
ATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTGCGCCCT  
TATTCCTTTTTCGCGCAT  
TTGCTTCTGTTTTTGTCTCACCAGAAACGCTGGTGAAAGTAAAGATGCTGAAGATC  
AGTTGGGTGCACGAGTGGGT  
ACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTTCGCCCCGAAGACGT  
TTTCCAATGATGAGCACTTT  
AAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTGACGCCGGGCAAGAGCAACTCGGT  
CGCCGCATACACTATTCTCA  
GAATGACTTGGTTGAGTACTCACCAAGTCACAGAAAAGCATCTTACGGATGGCATGACAG  
TAAGAGAATTATGCAGTGCTG  
CCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGA  
AGGAGCTAACCGCTTTTTTG  
CACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCC  
ATACCAAACGACGAGCGTGA  
CACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT  
TACTCTAGCTTCCCGCAAC  
AATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCCTTCTGCGCTCGGCCCTT  
CCGGCTGGCTGGTTTATTGCT  
GATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGTATCATTGCAGCACTGGGGCCAGAT  
GGTAAGCCCTCCCGTATCGT  
AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTG  
AGTAGGTGCTCTACTGATTA  
AGCATTGGTAATGTACAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACT  
TCATTTTAAATTTAAAGGATC

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## FIG. 6E

TAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTT  
CCACTGAGCGTCAGACCCCGT  
AGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGC  
AAACAAAAAACACCGCTAC  
CAGCTGGTTTGTTCGCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTC  
AGCAGAGCGCAGATACCAA  
TACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACC GCC  
TACATACCTCGCTCTGCTAA  
TCCTGTTACCAAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAA  
GACGATAGTTACCGGATAAG  
GCGCAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACC  
TACACCGAACTGAGATACCT  
ACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC  
GGTAAGCGGCGAGGCTCGAA  
CAGGAGAGCGCACGAGGGAGCTTCAGGGGGAAACGCCTGGTATCTTTATAGTCTGTGCG  
GGTTTCGCCACCTCTGACTT  
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC  
GCGGCCCTTTTACGGTTCCT  
GGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCTGCGTTATCCCCTGATTCTGTGGA  
TAACCGTATTACCGCCTTG  
AGTGAGCTGATACCGCTCGCCGAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGG  
AAGCGGAAGAGCGCCTGATG  
CGGTATTTTCTCCTTACGCATCTGTGCGGTATTTACACCGCATATGGTGCACTCTCAGT  
ACAATCTGCTCTGATGCCGC  
ATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCTATGGCTGCGCCCCGA  
CACC CGCCACACCCGCTGA  
CGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCC  
GGGAGCTGCATGTGTCAGA  
GGTTTTTACCGTCATCACGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGT  
CGTGAAAGCGATTACAGATG  
TCTGCCTGTTTATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTT  
CTGATAAAGCGGGCCATGTT  
AAGGGCGGTTTTTTCTGTTTGGTCACTTGATGCCTCCGTGTAAGGGGCAATTTCTGTT  
CATGGGGGTAATGATACCGAT  
GAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGG  
AACGTTGTGAGGGTAAACAAC  
TGGCGGTATGGATGCGGCGGGACCAAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCG  
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CCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCT  
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TTACGAAACACGGAAACCGAAGACCATTATGTTGTTGCTCAGGTGCGAGACGTTTTGC  
AGCAGCAGTCGCTTACGTTT  
GCTCGCGTATCGGTGATTATTCTGCTAACCAAGTAAGGCAACCCCGCCAGCCTAGCCGGG  
TCCGCTCTCCCTTATGCGAC  
TCTGCAATTAGGAAGCAGCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCGCAAGG  
AATGGTGCATGCAAGGAGAT  
GGCGCCCAACAGTCCCCCGGCCACGGGGCTGCCACCATACCCAAGCGGAAACAAGCGCTC  
ATGAGCCCGAAGTGGCGAG  
CCCGATCTTCCCATCGGTGATGTGCGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCC  
GGTGTGCGCGGCCACGATG  
CGTCCGGCGTAGAGGATCCCGCGAAATTAATACGACTCACTATAGG

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 97/00120

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: C 12 N 15/51; C 12 Q 1/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: C 12 N 15/51; C 12 Q 1/70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol.16, No.419 (C-981), 1992, Kokai No. 4-144 686 (KUNITADA SHIMOTOONO), abstract.	1
A	WO 92/08 734 A1 (CHIRON CORPORATION) 29 May 1992 (29.05.92), claims 1-6. -----	1

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

01 September 1997 (01.09.97)

Date of mailing of the international search report

05 September 1997 (05.09.97)

Name and mailing address of the ISA/ AT  
 AUSTRIAN PATENT OFFICE  
 Kohlmarkt 8-10  
 A-1014 Vienna  
 Facsimile No. 1/53424/535

Authorized officer

Wolf

Telephone No. 1/53424/436

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 97/00120

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